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(54) Title: ALPHA-1-ANTITRYPSIN AND ANTITHROMBINE-III VARIANTS

(57) Abstract

Serine protease inhibitors (serpins) are provided which: (a) are substantially incapable of inhibiting activated protein C; (b) do not require activation by heparin; and (c) comprise a target sequence capable of interacting with the proteolytic active site of thrombin thereby to inhibit the proteolytic activity of thrombin. Preferred serpins are muteins or variants of α_1-antitrypsin, modified by inclusion of a thrombin-specific target sequence derived from antithrombin-III (AT-III). Such serpins have the specificity and irreversibility of action of AT-III, but do not have to be co-administered with heparin.

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ALPHA-1-ANTITRYPSIN AND ANTITHROMBIN-III VARIANTS

1 Introduction

This invention relates to proteins having antithrombotic and/or anticoagulant activity, their preparation and uses of them.

2 Introduction to Serpins

Various physiological processes consist of a highly regulated network of proteases and their specific substrate (often termed cascades), examples of which include the coagulation, complement, plasminogen activation and inflammatory systems. Serine proteases are complemented by their serine protease inhibitors and these are vital for the regulation of proteolytic cascades in numerous mammalian species.

The family of SERine Protease INhibitors (abbreviated in the art to SERPINS) contains more than 40 members (Carrell, RW and Evans, DL, 1992, "Serpins: mobile conformations in a family of proteinase inhibitors", *Curr. Opin. Struct. Biol.* 2: 438-446). These were originally defined by their biological activity as protease inhibitors. However, the family was considerably expanded when a molecular structure, which was characteristic of all known serpins, was discovered. This allowed the categorisation of proteins that do not possess anti-protease activities, such as ovalbumin and protein Z, as serpins.

Serpins that do act as protease inhibitors do so by offering a loop of amino acids to the target protease as "bait". When the protease recognises its target sequence and cleaves the loop, the serpin undergoes a substantial

conformational change which "locks" it and the protease together. The essentially irreversible nature of this interaction, at least in the short-term, makes serpins especially potent inhibitors of proteases.

The overall structure of serpins has been known for some years and a number of detailed X-ray structures now exist. However, the exact mechanism and the structure of the amino acid "bait" loop has remained elusive because of the problems encountered with obtaining crystals of intact serpins.

The well characterised serpin α_1-antitrypsin (usually known as a1-AT or AAT in the art), which is an abundant plasma protein, is the major inhibitor of neutrophil elastase in man. The only structure that has been determined for AAT is that of the cleaved molecule (Figure 1) where the "trap" has been sprung and two of the amino acids of the bait loop, Met_{358} and Ser_{359}, are at opposite ends of the molecule. However, it is generally believed that the overall structure of AAT, apart from that of the reactive loop, is little changed by this event.

The current evidence as to how serpins work and how the bait loop, or reactive loop, is presented to the target protease is circumstantial. The postulated structure is derived from an analysis of the known structures of ovalbumin, an inactive but uncleaved serpin (Stein, PE, Leslie, AGW, Finch, JT and Carrell, RW, 1991, "Crystal structure of uncleaved ovalbumin at 1.95 Å resolution", *J. Mol. Biol.*, **221**: 941-959), and cleaved AAT (Löebermann, H, Tokuoka, R, Deisenhofer, J and Huber, RJ, 1984, *J. Mol. Biol.*, **177**: 531-556), shown schematically

in Figure 2. Extrapolation back to the probable structure of uncleaved AAT shows the "bait" loop as protruding from the top of the molecule (Figure 3). Here it is accessible to the target protease and effectively "primed" to spring back, and trigger the irreversible entrapment of the protease, on cleaving the labile bond.

3 Serpins as Therapeutic Agents

One of the aims of the invention is to provide new anticoagulants, especially for use in humans. These down-regulate the coagulation mechanism by inhibiting the activity of thrombin. Thrombin functions to promote coagulation by cleaving specific amino acid sequences from fibrinogen and hence promoting its self-polymerisation to form blood clots (Figure 4). Possible indications for the use of such new antithrombotic agents are: disseminated intravascular coagulation (DIC); thrombosis; myocardial infarction, unstable angina; thrombotic stroke; and pulmonary embolism.

Serpins with anticoagulant properties are well known and include antithrombin III (AT-III) and a natural variant of AAT. However the former of these has properties which make it less then ideal as a therapeutic agent and the latter is a potentially lethal mutation. Therefore, before going on to describe the invention, these two serpins will be discussed to define the sort of problems which the invention is designed to overcome.

3.1 Antithrombin III

Antithrombin III (AT-III) is a natural thrombin inhibitor found in the plasma. In view of this it can be regarded as a potentially useful therapeutic agent for coagulation disorders. However, it is only effective as an

inhibitor of thrombin in the presence of heparin. AT-III binds to heparin via a specific penta-saccharide recognition site. In turn, thrombin binds to the heparin molecule and then migrates along the oligosaccharide strand until it contacts, and is then specifically inhibited by, a correctly orientated molecule of AT-III. By this mechanism, the association constant of AT-III for thrombin is increased by more than an order of magnitude.

So the use of AT-III as a therapeutic agent requires the co-administration of heparin, which is itself a powerful anti-coagulant whose administration can lead to severe bleeding complications. AT-III/heparin treatment is therefore less reliable in its outcome than the use of a single active component and may not be convenient or appropriate for routine hospital use.

3.2 AAT Mutants

The study of the relationship between the structure and function of serpins began in 1978 with the investigation of a mutant of AAT having unexpected inhibitory activity (J.H. Lewis *et al*, "Antithrombin Pittsburgh: an α_1-antitrypsin variant causing haemorrhagic disease", *Blood*, 51:129 (1978)). A 14 year old boy having a severe, recurrent bleeding disorder was shown to have an abnormality in the AAT band on a serum protein electrophoresis gel. Analysis showed that the mutation was a Met to Arg substitution at position P1, which is at the N-terminal side of the reactive site of AAT (M. Owen *et al*, *The New England Journal of Medicine*, 309:694-698 (22nd September 1983)). This single amino acid substitution (Arg^{358}-P1) was sufficient to change AAT from being an inhibitor of the inflammatory enzyme human neutrophil elastase to being an inhibitor of the

procoagulant serine proteases thrombin, factors Xa, XIa and XII, plasmin, kallikrein and protein C.

This "Pittsburgh" mutant was identified as being of potential therapeutic use in the treatment of DIC, which results from the uncontrolled proteolytic activity of activated factors in the haemostatic cascade. It was hoped that it would afford protection because of its inhibition of factor Xa and thrombin. However, baboons with experimentally-induced Gram-negative sepsis, which provides a model of DIC, were found to die more quickly when given the Arg^{358} mutant of AAT than those given placebo. The result appears to be due to the large-scale inhibition of activated protein C. This explanation is supported by the observation that prophylaxis with hirudin, a specific inhibitor of thrombin, can protect the animals from thrombosis, but not from death; further, neutralising antibodies to protein C are toxic. The administration of protein C in conjunction with antithrombotics did, however, allow survival of baboons subjected to *E. coli* infusion (F.B. Taylor *et al*, *J. Clin. Invest.*, 79:918-925 (March 1987)). Another known mutant of AAT ($Ala^{357}Arg^{358}$) has also been found to inhibit the activity of activated protein C.

Protein C, in its activated state, is also a serine protease, and functions as an anticoagulant by inactivating two of the regulatory proteins of the coagulation pathway, factors Va and VIIa (see schematic diagram of the coagulation cascade, Figure 4). Protein C is activated by a complex of thrombin and the cell-surface thrombin binding protein thrombomodulin. Further information for this mechanism can be found in C.T. Esmon, *Science* 235:1348-1352 (13th March 1987). Suffice

it to say, though, that the administration of a serpin that significantly inhibits activated protein C can be fatal.

One other example of a serpin mutant which might be considered as an anti-coagulant is PAI-1. However, PAI-1, while potentially active against thrombin, is a strong inhibitor of tissue plasminogen activator. Plasminogen activators (t-PA and u-PA) are important in regulating the pathway for fibrin (and thus blood-clot) degradation via the powerful proteolytic enzyme plasmin. Removal of plasminogen activators would prevent the activation of plasminogen to plasmin and thus tip the coagulation balance in favour of clot formation. Therapeutically useful serpins should ideally have low activity towards the plasminogen activators.

Incidentally, a second reason for discarding PAI-1 as a potentially useful antithrombotic agent is that it spontaneously de-activates on short-term storage due to structural rearrangements. This low stability appears to be unique to PAI-1.

4 The Invention

The present invention seeks to ameliorate difficulties encountered with the use of known antithrombotics. It has been found that a thrombin-specific target sequence may be provided in a molecule which does not have the disadvantageous properties of AT-III.

According to a first aspect of the invention, there is provided a serine protease inhibitor ("serpin") protein which:

(a) is substantially incapable of inhibiting activated protein C;

(b) does not require activation by heparin; and

(c) comprises a target sequence capable of interacting with the proteolytic active site of thrombin thereby to inhibit the proteolytic activity of thrombin.

Serpins in accordance with the invention will consist essentially of a sequence of coupled amino acids, that is to say a plurality of residues joined by peptide bonds (-NHCO-). Such amino acids may include modified residues as well as the 20 residues found in nature, although in many embodiments they do not. The amino and/or carboxy terminus of the protein may be modified by the addition, substitution or deletion of organic groups. Serpin proteins of the invention are preferably made by recombinant DNA methods; they may be glycosylated if necessary, convenient or desirable.

Structure and Properties of Preferred Serpins of the Invention

A serpin of the invention comprises a target sequence, which interacts with the proteolytic active site of thrombin, and a protein base moiety. (The word "base" is not used as an antithesis to acid, but rather denotes the remainder of the serpin of the invention apart from the target sequence.) The target sequence acts as at least part of the serpin's reactive loop structure; the protein as a whole has serpin activity.

Serpins of the invention may be mutants, fragments or substantial homologues of natural molecules. In this specification, "mutants" or "mutations" refer either to

protein sequences which have been mutated by one or more additions, deletions or substitutions of amino acid residues, or to corresponding changes to the bases in the DNA sequences that code for the protein sequences. "Protein fragments" include molecules which are shorter than those from which they are derived and include functional fragments, in which an additional or unwanted portion of the molecule is masked or inactivated rather than being physically removed. A particular protein or nucleic acid sequence may be described as being "substantially homologous" to another: this means a degree of homology of at least 40%, 50%, 60% or preferably at least 70%. Mutants, fragments and homologues within the scope of the invention will generally have substantially the same relevant activity as the molecule with which it is being compared, in terms either of the level of activity or the specificity of activity or both.

5.1 The Reactive Loop Target Sequence of Serpins of the Invention

The target sequence inhibits thrombin by binding to the proteolytic active site (or active cleft as it is sometimes known); the result is that fibrinogen is prevented from being converted into fibrin.

The target sequence will usually be from 2 to 20 residues long. Since the proteins of the invention are preferably at least 300, 350 or 400 residues long, the target sequence itself will usually constitute only a relatively small part of the entire molecule.

The target sequence preferably comprises a sequence which is a reactive loop of a naturally occurring serpin, or a

fragment thereof, or is substantially homologous to such a sequence. Such a naturally occurring serpin is antithrombin-III (AT-III), at least part of whose reactive loop is preferred for use as a target sequence in serpins of the invention. Fragments, mutants and substantial homologues of reactive loops of other naturally occurring serpins are useful as target sequences in the present invention, provided that they too inhibit the proteolytic activity of thrombin.

The target sequence will be located in or on a serpin of the invention such that it is able to interact with the active site of thrombin; it may therefore form, or constitute part of, a loop structure in a serpin of the invention, preferably a loop that is at least partially exposed.

In the art, residues that make up or are near the reactive loop of a serpin are usually designated by the letter "P" followed by a number; this system will be adhered to in this specification. These designations can refer to residue positions in the target sequence, in the base moiety or in the whole serpin (or in some or all of them at the same time). The residues count down from the amino end towards the functional centre of the loop and then, with the additional prime (') designation, up again away from the centre, as follows (using arbitrary start and finishing residues):

...P5-P4-P3-P2-P1-P1'-P2'-P3'-P4'-P5'...

The neighbouring P1 and P1' residues are usually considered to be two of the most important in determining the level and spectrum of inhibitory activity. The precise definition of where the reactive loop starts and

finishes, and so which residues are included, seems to vary, but the loop can generally be thought of as spanning residues P17 to P10', P11 to P5' or P8 to P2', in increasing order of specificity.

In preferred embodiments of the invention, the target sequence comprises a functional target sequence contained in the reactive loop of AT-III, such as Gly-Arg-Ser. These three amino acids correspond to the P2-P1-P1' residues of the reactive loop of AT-III, and preferably replace the corresponding residues in the molecule which provides the protein base moiety.

Particularly preferred target sequences comprise:

Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-Pro-Arg-Ser-Leu-Asn

or a fragment, homologue or mutant thereof; or

Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-Gly-Arg-Ser-Leu-Asn

or a fragment, homologue or mutant thereof. This latter sequence is the active site present in the thrombin inhibitor AT-III, and spans residues P10 to P3'.

5.2 The Base Molecule of Serpins of the Invention

The base protein moiety can be any suitable polypeptide sequence, but preferably it comprises sequences of a natural serpin. This is advantageous because of the irreversible nature of serpin inhibition. A short peptide analogue of the AT-III reactive loop, for instance, would only be a competitive inhibitor and would therefore be needed at much higher concentrations to give the desired biological effect. A second advantage of using a large molecule like a serpin, with a molecular mass in the range of 45kDa, is that it would have a much longer half-life in the circulation compared with smaller molecules which would be cleared by glomerular filtration.

The target sequence may conveniently be positioned in, close to or, preferably, in place of at least part of an active loop of a serpin from which the base moiety is derived. While the base moiety may comprise all of the serpin from which it is derived (apart, of course, from any residues being substituted by other residues to form the target sequence), it does not have to do so: certain residues or domains of the base moiety serpin may be unnecessary or undesirable, and other mutations may be introduced elsewhere in the molecule. However, enough of the base moiety serpin should for preference be present to ensure an essentially irreversible inhibition of thrombin.

A particularly favoured serpin is α_1-antitrypsin (AAT). Preferred serpins of the invention are therefore AAT-derived molecules in which the reactive loop has been modified to generate a target sequence which is capable of interacting with the proteolytic site of thrombin.

In AAT, the modifications made to the molecule may encompass one or more residues in the region from P11 to P5', for example from P10 to P3'. The AAT molecule may additionally be modified or mutated at other places: AAT-based embodiments of the invention will for choice be based on the naturally occurring sequence of AAT, but they do not have to be. Both mutants and fragments of AAT are also contemplated.

AAT is particularly suitable for modification because of the functional neutrality of most of the molecule: the interaction of AAT with its target protease occurs primarily between AAT's reactive loop and the active cleft of the protease. The function of AAT is best

defined by its reactive loop, while in some other serpins secondary specificity domains are required to determine the spectrum of inhibition.

By using AAT as the base molecule, it is readily possible to create a thrombin inhibitor that does not require activation by heparin. This is so even though the target sequence may, as is preferred, be derived from AT-III, which does require heparin as a co-factor.

Preferred serpins of the invention in which AAT provides the base moiety and which include a target sequence derived from at least part of the reactive loop of AT-III are as follows. Residues which are the same as in AAT are indicated by a dash. The corresponding residues in AT-III are given at the bottom for ease of reference.

	P-12	*11*	*10*	*9*	*8*	*7*	*6*	*5*	*4*	*3*	*2*	*1*	*1'*	*2'*	*3'*	*4'*	*5'*	*6'*	*7'-P'*
AAT	**A**	**A**	**G**	**A**	**M**	**F**	**L**	**E**	**A**	**I**	**P**	**M**	**S**	**I**	**P**	**P**	**E**	**V**	**K**
Loop Swap I	-	-	A	S	T	A	V	V	I	A	G	R	S	L	N	-	-	-	-
Loop Swap II	-	-	-	-	-	A	V	V	I	A	G	R	S	L	-	-	-	-	-
Loop Swap III	-	-	-	-	-	A	V	V	I	A	G	R	S	L	N	-	-	-	-
LS-Pro	-	-	-	-	-	A	V	V	I	A	P	R	S	L	N	-	-	-	-
P' Swap 1	-	-	-	-	-	-	-	-	-	-	-	R	S	L	N	-	-	-	-
P' Swap 2	-	-	-	-	-	-	-	-	-	-	-	R	T	L	L	-	-	-	-
P3' Asn	-	-	-	-	-	-	-	-	-	-	-	R	-	-	N	-	-	-	-
P3' Lys	-	-	-	-	-	-	-	-	-	-	-	R	-	-	K	-	-	-	-
P3' Arg	-	-	-	-	-	-	-	-	-	-	-	R	-	-	R	-	-	-	-
AT-III	**A**	**A**	**A**	**S**	**T**	**A**	**V**	**V**	**I**	**A**	**G**	**R**	**S**	**L**	**N**	**P**	**N**	**P**	**N**

Serpins of the invention are substantially incapable of inhibiting activated protein C. Additionally, they are preferably substantially incapable of inhibiting tissue plasminogen activator (t-PA), either. By "substantially incapable" it is meant that, at pharmacologically useful concentrations of a serpin of the invention, there is no significant interference with the other molecule's relevant activity and/or function.

5.3 Modified ATIII

Alternatively, it is possible to modify the loop sequence of ATIII to create a modifed ATIII which has the properties described above, and particularly a form which does not require the presence of heparin to exhibit antithrombin activity. Such a modifed ATIII comprises the sequence:

P-7	6	5	4	3	2	1	1'	2'	3'
A	V	V	I	A	P	R	S	L	N

5.4 Kinetic Properties of Serpins of the Invention

Although serpins may associate only slowly with a protease, they may nevertheless remain tightly bound. Since most serpins bind almost irreversibly, it is best to compare how effective different serpins are on the basis of their association constants (k_{ass}). Low association constants mean that the serpin binds slowly.

Some specific values for the activity of the serpins of the invention will now be given. Such proteins preferably have one or more (and preferably all) of the following association rate constants (k_{ass}, in $M^{-1}s^{-1}$):

1. with thrombin: at least 10^3, preferably at least 10^4, and optimally at least 5×10^4;

2. with activated protein C: no more than 10^3, preferably less than 10^2, and optimally below 10;

3. with tPA: less than 1.4×10^2.

Preferred serpins of the invention have an antithrombotic activity of at least 1000 times, or even at least 1500

times, as much as non-mutated naturally occurring (i.e. wild-type) AAT.

Although certain preferred features and characteristics of serpins of the invention have been described above in relation to particular embodiments, those features and characteristics are, where appropriate, also applicable to other serpins of the invention.

6 Other Aspects of the Invention

A second aspect of the invention relates to a protein of the first aspect for use in medicine, for example as an antithrombotic agent.

A third aspect of the invention relates to a pharmaceutical or veterinary composition comprising one or more proteins of the first aspect and a pharmaceutically or veterinarily acceptable carrier or excipient.

Such compositions may be adapted for intravenous administration and may therefore be sterile. Included in this aspect of the invention are sterile preparations of protein(s) of the first aspect in isotonic physiological saline and/or buffer. Compositions of the invention may additionally comprise, if required, one or more anticoagulant, antithrombotic and/or thrombolytic agents, for example hirudin and/or t-PA or fragments or mutants thereof and/or Streptokinase.

The protein may be supplied in unit dosage form, for example as a dry powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of protein in activity

units. These units may, for example, be AT-III units (ATUs), if the protein has antithrombotic activity. If the protein is to be administered by infusion, it may be dispensed by means of an infusion bottle containing sterile water (for injection) or saline. Where it is to be administered by injection, it may be dispensed by an ampoule of water (for injection) or saline. The infusible or injectable composition may be made up by mixing the ingredients prior to administration.

The amount of protein to be administered will depend upon the effect required, such as the amount of thrombosis, the required speed of action, and the seriousness of the condition of the patient to be treated (for example in both the number and size of clots). The precise dose to be administered, because of the very nature of the condition which the proteins one intended to treat, will be determined by the physician. As a guide, however, in antithrombotic treatment a patient being treated may generally receive a daily dose of from 0.1 to 10 mg/kg of body weight, such as from 3 to 7 mg/kg either by injection or by infusion.

A fourth aspect of the present invention relates to a process for the preparation of a pharmaceutical or veterinary composition of the third aspect, the process comprising admixing one or more proteins of the first aspect with a pharmaceutically or veterinarily acceptable carrier or excipient.

A fifth aspect of the invention relates to the use of one or more proteins of the first aspect in the preparation of an antithrombotic or anticoagulant agent. The protein may also be used to treat DIC (disseminated intravascular

coagulation), unstable angina, myocardial infarction, thrombotic stroke, thrombosis, pulmonary embolism, and other blood clotting disorders. The agent can be used in treatment or prophylaxis or both.

The invention has application in a method of treatment or prophylaxis of thrombosis (or any of the other conditions or disorders mentioned in relation to the fifth aspect), the method comprising administering to a subject an effective amount of one or more proteins of the first aspect or a composition of the third aspect.

A seventh aspect of the invention relates to a process for the preparation of a protein of the first aspect, the process comprising coupling successive amino acid residues and/or oligo- and/or polypeptides together. This may be achieved by chemical synthesis, but is preferably by translation of a nucleic acid *in vivo* or even *in vitro*. Preferred serpins of the invention, which are based on a natural serpin, may be constructed using established techniques of recombinant DNA technology, including site-directed mutagenesis.

Such nucleic acids are themselves part of the invention, according to an eighth aspect of which there is provided nucleic acid (which may be RNA or, preferably, DNA) which:

(a) has a sequence coding for a protein of the first aspect, or its complementary strand;

(b) is substantially homologous with, or hybridises under stringent conditions to, either of the sequences in (a); or

(c) would be substantially homologous with, or would hybridise under stringent conditions to, a sequence in (a), but for the degeneracy of the genetic code.

Suitable stringent conditions include salt solutions of approximately 0.9 M at temperatures of from 35 to 65°C.

Nucleic acid of the invention may be recombinant and/or isolated nucleic acid. The nucleic acid may be in the form of a vector, such as a plasmid, phagemid, cosmid or virus, and in some embodiments contains elements to direct expression of the protein, for example in a heterologous host. Non-expressible vectors are useful as cloning vectors.

Constructs of nucleic acids, which need not be vectors, include transgenic expression constructs for creating transgenic (non-human) animals that express the proteins of the first aspect.

A ninth aspect of the invention relates to a host cell transformed or transfected with a vector of the ninth aspect. Host cells may be prokaryotic or eukaryotic and include mammalian cells, insect cells, yeasts (such as *Saccharomyces cerevisiae*) and bacteria (such as *Escherichia coli*).

A tenth aspect of the invention relates to a process for the preparation of nucleic acid of the eighth aspect, the process comprising coupling successive nucleotides and/or ligating oligonucleotides and/or polynucleotides together. The nucleic acid may be synthesised chemically, although it is preferred to use a nucleic

acid-directed polymerase, preferably *in vivo*. For introducing mutations, the nucleic acid may be suitably modified using site-directed cassette and/or PCR mutagenesis.

A eleventh aspect of the invention relates to a process for the preparation of a protein of the first aspect, the process comprising culturing a host of the ninth aspect under conditions that allow expression of nucleic acid encoding the protein.

A twelfth aspect relates to the use of a protein of the first aspect as a diagnostic agent, for example as a standard in an assay.

A thirteenth aspect relates to an assay for a serine protease, for example thrombin, the assay comprising the use, as a standard, of a detectably labelled protein of the first aspect for determining or detecting the presence of the serine protease in a sample. Such an assay can be a competitive assay.

A fourteenth aspect relates to a diagnostic kit comprising at least one container comprising an amount, for example a known amount, of a detectably labelled protein of the first aspect.

An fifteenth aspect of the invention relates to a non-human animal containing (e.g. recombinant) nucleic acid of the eighth aspect. Preferably the animal is an avian species or a placental mammal: the nucleic acid can be comprised in the transgene. Such a mammal may have a germline which includes the nucleic acid, adult females of the line being capable of expressing a protein of the

first aspect in the mammary gland so that it accumulates in the milk, from which the protein may be recovered. Preferred mammals include cows, sheep, goats, mice, rabbits, camels and pigs.

The mammary gland expression system has the advantages of high expression levels, low cost, correct processing and accessibility. Known proteins, such as bovine and human α-lactalbumin, have been produced in lactating transgenic animals by several workers (Wright *et al*, *Bio/Technology* **9** 830-834 (1991); Vilotte *et al*, *Eur. J. Biochem.* **186** 43-48 (1989); Hochi *et al*, *Mol. Reprod. and Devel.* **33** 160-164 (1992); Soulier *et al*, *FEBS Letters* **297**(1,2) 13-18 (1992)) and the system has been shown to produce high levels of protein.

Other transgenic animals, including transgenic mammals expressing heterologous protein in the milk of adult females, have been described in the literature. For example, WO-A-8800239 and WO-A-9005188 describe the production of transgenic mammals such as sheep which express pharmaceutically valuable proteins including factor IX and AAT. The methodology set out in these and other publications may be adapted to produce transgenic animals in accordance with the present invention. It is preferred to use known dairy mammals, such as cows, sheep, goats, rabbits, mice, camels or pigs, as hosts in the present invention, but the choice of host will be dictated primarily by convenience and not by any requirement of the invention itself.

Transgenic animals or other hosts according to the invention may be prepared by any convenient methodology. Accordingly, the invention is not limited to any

particular method for their preparation. Transgenic animals, for example, may be prepared by microinjection, as described in WO-A-8800239 and WO-A-9005188 above, or they may be prepared by embryonic stem cell technology such as is described in WO-A-9003432, or by any other known methods.

Preferred features and characteristics of each aspect of the present invention are as for each other aspect *mutatis mutandis*.

7. Drawings

The accompanying drawings, some of which have already been referred to and some of which are relevant to the examples which follow, relate to preferred embodiments of the invention. In the drawings:

FIGURE 1 shows the structure of α_1-antitrypsin (AAT);

FIGURE 2 shows, schematically, cleaved AAT;

FIGURE 3 shows the probable structure of uncleaved AAT shows the "bait" loop as protruding from the top of the molecule;

FIGURE 4 shows a schematic diagram of the coagulation cascade;

FIGURE 5 shows the construction of the plasmid vectors pTermAT and p_{Δ}N15;

FIGURES 6 and 7 are maps of the plasmid pCATh;

FIGURES 8 and 9 are maps of the plasmid pATh;

FIGURE 10 is a map of the plasmid pTermAT; and

FIGURE 11 is a print of a sequencing gel confirming the sequence of the mutant in Example I-H2 below.

8. Examples

I. GENERAL MATERIALS AND METHODS

A. Bacterial strains and expression

The *Escherichia coli* strains TG2 (supE hsdΔ5 thi Δ(lac-proAB) Δ(srl-recA)::Tn10(tet^r) F'[traD36 proAB$^+$ lacI^q lacZΔM15]) and the B strain BL21 (F$^-$ ompT r^-_Bm^-_B hsdS gal lon-) DE3 (λcIts857 ind1 Sam7 nin5 lacI lacUV5-T7 gene 1) were in culture in the laboratory. *E. coli* BL21 (DE3) was obtained from Dr. F. William Studier at Brookhaven National Laboratories, Upton, New York, USA. Other strains may be substituted.

B. Media and plates

The following types of liquid media, agar and agarose plates were prepared: bacterial 2 x TY medium (containing 16 g/l bacto-tryptone, 10 g/l bacto-yeast extract and 5 g/l NaCl), M9 bacterial minimal medium (containing 64 g/l $Na_2HPO_4.7H_2O$, 15 g/l KH_2PO_4, 2.5 g/l NaCl, 5 g/l NH_4Cl and 0.4% glucose).

Agar and agarose plates were prepared by adding agar or agarose to 1 litre of liquid medium. The following were used during the course of this work: bacterial TYE plates (containing 10 g/l tryptone, 5 g/l bacto-yeast extract, 8 g/l NaCl and 15 g/l agar) were supplemented with ampicillin (50 - 100 μg/ml) when selecting for plasmids which conveyed antibiotic resistance, bacterial minimal plates (100 ml/l 10x M9 salts, 1 ml/l 1 M $MgSO_4$, 2 mg/l vitamin B1, 20 g/l D-glucose {added after

autoclaving}), yeast YPD plates (11 g/l yeast extract, 22 g/l bacto-peptone, 55 mg/l adenine sulphate, 22 g/l agar, 10 g/l D-glucose {added after autoclaving}) and yeast minimal medium plates (22 g/l agar in yeast minimal medium).

C. Column Chromatography

Chromatography was performed on a WATERS™ 650E Advanced Protein Purification System with a WATERS™ 480 variable wavelength detector (Harrow, UK). Resins were packed in PHARMACIA™ XK series of columns. Resins used were: PAE-300 (Amicon, Danvers, MA, USA), and Chelating SEPHAROSE™ (Pharmacia, Uppsala, Sweden).

Purification of oligonucleotides was performed on a GEN-PAK FAX™ column (Waters) on a Waters HPLC system.

D. Chemicals

Trizma base, ATP, X-gal, and IPTG were purchased from Sigma (Poole, UK). SEQUENASE™ kits were purchased from USB (Cleveland, Ohio, USA). GENECLEAN™ and MERMAID™ kits were supplied by Stratech (Luton, UK). Ultrapure enzyme grade urea, agarose, LMP-agarose and caesium chloride were from BRL (Gaithersburg, USA). Deoxy- and dideoxynucleoside triphosphates were from Pharmacia (Uppsala, Sweden). X-ray film was purchased from Fuji (Tokyo, Japan). Ampicillin, kanamycin and chloramphenicol were obtained from Sigma (Poole, UK). Rifampicin was obtained as RIFAMPIN™ from Marion Merrell Dow, (Uxbridge, Middx.).

p-Nitrophenylguanidinobenzoate and polyclonal rabbit antibodies against human AAT were obtained from Sigma (Poole, UK). ANTIFOAM™ 289 was purchased from Sigma UK (Poole, UK). S-2266, S-2238, S-2302 and S-2222 were

purchased from Chromogenix, Molndahl, Sweden. PEFACHROM™-t-PA was purchased from Pentapharm, Basel, Switzerland.

All other chemicals were purchased from BDH (Dagenham, UK) and were of biological, AnalR or Electran grade.

E. Equipment

Submerged agarose gel and vertical gel apparati were supplied by the University of Edinburgh Clinical School workshop. DNA and RNA were visualised with a short wavelength UV trans-illuminator from UVP (San Gabriel, USA). PCR amplifications were performed on a PERKIN ELMER CETUS™ (Beaconsfield, UK) DNA thermal cycler. Electroporation was performed with GENEPULSER™ equipment purchased from Bio-Rad. The hybridisation oven, model HB-1, was a gift from Techne (Duxford, Cambridge, UK).

F. Enzymes

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and T4 polynucleotide kinase were supplied by New England Biolabs unless otherwise stated (distributed by CP Laboratories, Bishop's Stortford, UK). *Taq* (from *Thermus aquaticus*) thermostable DNA polymerase was purchased from Promega Ltd. (Southampton, UK). Calf intestinal phosphatase and nucleotide triophosphates were supplied by Boehringer Mannheim (Lewes, UK). DNase-free RNase A (bovine pancreas), DNaseI (bovine pancreas) and Proteinase K (from *Tritrachium album*) were from Sigma.

Thrombin and factor Xa were purified from plasma by Dr. Stuart R. Stone. Trypsin and plasmin were purchased from Sigma (Poole, UK) and activated protein C was a gift of Dr J. Stenflo and Dr A. Ohlin.

G. DNA

pTZ18R was obtained from Pharmacia (Upsalla, Sweden); M13K07 was obtained from Avron (Department of Haematology, Cambridge); M13mp9a1-AT was obtained from F.Reeve (Department of Haematology, Cambridge); pMAX20 was supplied by F. Hill (Dept Haematology, University of Cambridge); and pLysS and pET8c were provided by F. William Studier, Brookhaven National Laboratories, Upton NY, USA. Molecular weight markers *Hin*dIII-digested λDNA and *Hae*III-digested ϕX174 DNA were obtained from New England Biolabs (Beverly MA, USA); 123bp and 1Kb ladders were obtained from BRL (Gaithersburg MD, USA). M13mp9 phage, into which the α_1-antitrypsin cDNA was cloned to form M13mp9a1-AT, is available from many suppliers (such as Pharmacia). α_1-antitrypsin cDNA was obtained from Dr. Richard Foreman at Southampton University, Southampton, Hampshire. pMAX20 is used solely as a source of the par locus, which is originally from pSC101.

All oligonucleotides were synthesised by the Department of Biochemistry, University of Cambridge, and were supplied as lyophilised preparations containing approximately 0.2μmoles of deprotected oligonucleotide. The oligonucleotides comprising the portable translation initiation site were purchased from Pharmacia.

H. Construction of Expression Vectors

pTermAT and pΔN15, vectors designed for the expression of a1-AT and mutants thereof in *E. coli* were constructed as illustrated in Figure 5. All enzymes were used according to the manufacturers' instructions.

The vectors contain the origin of replication and ampicillin resistance gene derived from pUC. They also

contain the f1 origin of replication for the purpose of generating single-stranded DNA. The coding sequence for α_1-antitrypsin (AAT) is under the control of a T7 RNA polymerase promoter and a synthetic ribosome binding site. The sequence coding for AAT is followed by a terminator of RNA transcription derived from pET8c and a par locus derived from pSC101 (via pMAX20).

The coding sequence for AAT may have one or more codons deleted from the 5' end of the gene for the purposes of improving expression in *E. coli* or convenience of cloning; there may also have other mutations within the gene for the purpose of generating restriction sites or for the purpose of generating mutant proteins.

All routine molecular biology protocols were obtained from Sambrook J., Fritsch F. and Maniatis T. "Molecular Cloning: A Laboratory Manual". Cold Spring Harbor Laboratory Press, Second Edition (1989). Protocols for sequencing DNA were from the manufacturers of SEQUENASE™ (USB, Cleveland, Ohio, USA). A few specific protocols used in the construction of the vectors are now described.

H1. Generation of a new Pst1 site

Three primers were used in this procedure:

Primer 1 (5'-ACTGAAGCTGCAGGGGCCAT-3') contains the mutation (underlined) necessary to create the *Pst*I site by silent mutagenesis;

Primer 2 (5'-ATACCGCTCGAGTTATTTTTGGGTGGG-3') anneals to the 3' end of the AAT gene and on the opposite strand to Primer 1; and

Primer 3 (5'-ATACCGCTCGAGATCAAACGGGAGGATCCCCAGGGA-3') anneals to the 5' end of the AAT gene.

To generate pCath (see Figures 6 and 7), the *Pst*I site in pATh (see Figure 8) was first removed by digesting the plasmid with *Pst*I and blunted back with T4 DNA polymerase as recommended by the manufacturers (New England Biolabs). The resultant DNA was then circularised by ligation, and the products screened with *Pst*I for the loss of that site.

A new *Pst*I site was then created in a position bordering the reactive site loop by silently mutating one of the bases coding for the P10 amino acid. This was done by the technique known as 'megaprimer mutagenesis'. For the first PCR reaction Primer 1 and Primer 2 were used. The PCR reaction was performed in the presence of 25 p moles of each of these two primers, 10mM of each dNTP, 100ng of pATh and 5 units of *Taq* polymerase, in buffer supplied by the manufacturers of *Taq*; 30 cycles were performed (94°C for 20s, 50°C for 30s and 74°C for 20s). The product of this reaction was a 270bp piece of DNA, which was purified by electrophoresis on a 2.5% low melting point agarose gel. The DNA containing agarose was then melted at 50°C for 20 minutes, and used directly as a primer in the second PCR reaction.

A second PCR reaction was then performed, using 10µl of the above preparation as one primer, 25 nmoles of Primer 3 as a second primer, 10mM of each dNTP, 100ng of pATH, 5 units of *Taq* polymerase, in buffer supplied by the manufacturers of *Taq*. The PCR reaction was performed for 30 cycles (94°C for 20 s, 50°C for 30 s and 74°C for 60 s).

The product of this reaction was a small amount of DNA corresponding in size to the AAT gene, which was purified by electrophoresing on a 0.8% LMP agarose gel in TAE buffer. The band was excised and the agarose melted as previously described; 10μl of this was used as a template in a final PCR reaction employing oligonucleotides Primer 2 and Primer 3, each at 25 nmoles, 10mM of each dNTP, 5 units of *Taq* polymerase, in the buffer recommended by the manufacturers of *Taq*. The PCR reaction was performed for 30 cycles (94°C for 20 s, 50°C for 30 s and 74°C for 60s). The product of this PCR reaction is the full-length AAT coding sequence which has a silent mutation creating an internal *Pst*I site. This DNA was then digested with *Bst*XI and *Ava*I as recommended by the manufacturers (New England Biolabs), as was the pATh vector which had had its *Pst*I site removed (see above). The cut DNAs were gel-purified and the mutant ~250bp fragment containing the *Pst*I site was cloned into pATh to yield pCATh (see Figure 9).

The par locus and terminator of RNA transcription were cloned into pCATh as shown in Figure 10 to yield pTermAT. 15 codons (45 nucleotides) were then deleted from the 5' end of the al-AT coding sequence as shown to yield pΔN15. Briefly, pTermat was grown in *E. coli* JM110 to avoid methylation problems with *Bcl*I. The plasmid DNA was then completely digested with *Bcl*I and partially digested with *Bam*HI. The products were purified on an agarose gel. The nearly full length fragment, which has had the 45 base pair fragment at the beginning of the gene removed, was isolated from the gel and circularised by ligation to give pΔN15. At each stage, screening for correct recombinants was performed by restriction analysis.

H2. Mutagenesis of pTermAT to make a 'Loop-Swap' mutant

The mutagenic oligonucleotide used, designated Primer 4, was as follows:

5'-TCGGGGTTTAGCGAACGGCCAGCAATCACAACAGCGGTACTTGCTGCA-3'.

This results in a DNA molecule which codes for AAT except that residues P10 to P3' inclusive are now identical to those found in AT-III.

The method described below and illustrated in Figure 5 allows cassette mutagenesis of the AAT with the use of only one mutagenic oligonucleotide. The oligonucleotide is complementary only to the overhang of the cut *Pst*I site and was ligated to the vector cut with *Pst*I. The product of the ligation was then used as a template in a PCR reaction using Primer 3 and Primer 4. A DNA molecule was thereby generated which has the mutagenic sequence grafted onto the end of an incomplete AAT-coding sequence. This was then used to generate a new coding sequence, coding for an AAT Loop-Swap protein, as shown in Figure 5. The resultant molecule was sequenced, confirming the introduction of the mutations (Figure 11).

II. EXPRESSION AND PURIFICATION OF AAT IN *E. COLI*

A. Fermentation of wild-type and mutant AATs

E. coli strain BL21 (DE3) transformed with pTermAT was grown in a 20 litre fermenter (LSL Biolafitte S.A., St. Germain-en-Laye, France) containing 10 litres of medium. The medium used contained, per litre: 11.25g K_2HPO_4, 1.25g KH_2PO_4, 1.25g NaCitrate, 2.5g $(NH_4)_2SO_4$, 12.5g casamino acids, 6.25g yeast extract. 1ml of ANTIFOAM™ 289 (Sigma, Poole, UK). This medium was autoclaved *in situ* and adjusted to pH7.0 with NaOH. A 500ml fresh overnight culture was used as an inoculum. During the inoculation, the following was added: 20ml 1 M $MgCl_2$, 20ml 50% glucose and 10ml 0.1g/ml ampicillin. The growth and production

phases were performed at 37°C with a rotor speed of 200-400 rpm. pH was regulated automatically and adjusted with 12.0M NaOH. Biomass was monitored by OD_{600} and growth rate by percent saturated oxygen. When the culture had reached an OD_{600} of 4.0, the fermentation was fed with a solution of 25% glucose, 10% yeast extract and 10% casamino acids at 2.0ml/min. The T7 RNA polymerase gene resident in the DE3 lysogen was induced when the OD_{600} had reached 10.0 by adding 1.0g of IPTG. After 30 further minutes of growth, the *E. coli* RNA polymerase was selectively inhibited by the addition of 10ml of 0.1 g/ml rifampicin dissolved in dimethylsulphoxide (final concentration 100 μg/ml; this step was omitted for pΔN15. The production phase was allowed to proceed for a further 3 hours, after which the cells were harvested by repeated centrifugation 'pellet-on-pellet' at 6000g for 20 min. The cell paste thus obtained was stored at -70°C until purification was carried out.

B. Purification of fermented AAT

All purification steps were carried out at 4°C. The frozen cells (approximately 100g) were thawed in an equal quantity (v/w) of 300 mM NaCl, 50 mM Tris-HCl pH 8.0, 5mM EDTA, 1mM PMSF, 1mM β-mercaptoethanol, and disrupted by two passages through a French press (Aminco, Urbana, II, U.S.A.). Polyethylene glycol (M_r 8000) was added to the lysate to a final concentration of 8% (w/v), and after 1 hour at 4°C the cell debris was removed by centrifugation at 10,000 x g for 1 hour. Further polyethylene glycol (M_r 8000) was added to the supernatant to a final concentration of 28% (w/v), and after stirring for 1 hour at 4°C a second centrifugation was performed. The pellet was dissolved in 50 ml of 20 mM sodium phosphate, pH 6.8 containing 5 mM EDTA, 1mM PMSF and 1 mM

β-mercaptoethanol. This was subjected to a further centrifugation to remove undissolved material and filtered through a 0.2μm filter (ACRODISC™) before loading onto a 2.6cm x 20 cm PAE-300 column equilibrated with 20mM sodium phosphate pH6.8, 1mM β-mercaptoethanol. The AAT was eluted with a 1 litre linear gradient of 0 - 250 mM NaCl in the equilibration buffer. The eluted fractions were analysed by rocket immuno-electrophoresis using polyclonal anti-AAT antibodies. Those fractions richest in AAT were pooled; then loaded onto a 1.6 cm 15cm column of Zn^{2+}-charged Chelating SEPHAROSE™ equilibrated in 20 mM Tris-HCl pH 8.0, 150mM NaCl. The AAT was eluted with a linear gradient of 0 - 75mM glycine in the equilibration buffer. Fractions which contained AAT, as determined by rocket immuno-electrophoresis, were analysed by SDS-PAGE on a 10% acrylamide gel. Those fractions that were over 95% pure were pooled; polyethylene glycol (M_r 6000) was added to 0.1%, EDTA to 1 mM and β-mercaptoethanol to 0.5mM. Aliquots of 1ml were frozen in liquid nitrogen before being stored at -70°C.

III. KINETIC ASSAYS

All kinetic assays were performed at 37°C in 30mM sodium phosphate buffer pH 7.4, containing 160mM sodium chloride, 0.1% polyethylene glycol (M_r 6000) and 0.2mg/ml of bovine serum albumin.

A. Titrations

Trypsin and thrombin were titrated with *p*-nitrophenyl *p'*-guanidinobenzoate in 0.1M sodium barbitone/0.02M $CaCl_2$ pH 8.3 at 25°C, as described (Chase, T. and Shaw, E. *Methods Enzymol*. 19 20-27 (1970)).

The concentrations of the serpins were determined by titrating with the above enzymes. The enzyme was incubated with varying amounts of inhibitor at a suitable concentration range; after an appropriate incubation time for allowing the full formation of the enzyme-inhibitor complex, the amount of residual enzyme activity was determined by adding buffer and substrate. Linear regression analysis of the residual enzyme activity plotted against amount of serpin added enabled the determination of the serpin concentration. The concentrations of the remaining enzymes was determined by titrating against the serpin as described above.

B. Determination of K_m Values

Initial rates for the hydrolysis of chromogenic substrates by the various enzymes used were determined with a range of different substrate concentrations. The data were fitted to the Michaelis-Menten equation by non-linear regression. The K_m values of thrombin with S-2266 and S-2238 were 262 and 2.1μM respectively. The K_m value of trypsin with S-2302 was 91μM, of factor Xa with S-2222 was 472μM, of plasmin with S-2302 was 300μM, of activated Protein C with S-2266 was 654μM, and of t-PA with PEFACHROM™-tPA was 345μM. Substrate concentrations were determined from their absorbance at 342nm by using a molar absorption coefficient of 8270 (Heeb *et al*, *J. Biol. Chem.* **265** 2365 (1990)).

C. Determination of Kinetic Parameters by Progress Curve Kinetics

The reactions were started by adding enzyme to the cuvettes. Five to seven assays were performed together: one cuvette without inhibitor, and the rest with different inhibitor concentrations. The absorbance at

400-410nm was measured every 15 to 30 seconds for between 1 and 8 hours, using a HEWLETT PACKARD™ 8452A diode-array spectrophotometer. Control assays in the absence of inhibitor indicated that the enzymes were stable for the time of the assay in the buffer used. In general, data were used only if the level of substrate utilisation was less than 5%. The ranges of substrate concentrations used were between 100 and 200μM.

$$EI \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} E + S \overset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

$$K_i = k_{off}/k_{on}$$

Scheme I

The inhibitory mechanism for the inhibitor-enzyme combinations used is shown by Scheme I, in which E, I and S represent the enzyme, inhibitor and substrate, respectively. The association constant (k_{on}) and the dissociation rate constant (k_{off}) are related to the inhibition (dissociation) rate constant (K_i) for the complex by the relationship given in the scheme. Progress curve data were fitted to the equation that describes this mechanism by non-linear regression (Stone *et al*, *Biochemistry* 25 4622 (1986)) to yield values for the apparent association rate constant (k_{on}') and apparent inhibition constant (K_i'). True values for these constants were calculated by correcting for the concentrations of substrate used in the experiments by using the following expressions:

$$K_i = K_i'/(1+[S]/K_m)$$
$$k_{on} = k_{on}'(1+[S]/K_m)$$

D. Kinetic Data

Loop-Swap Pro (LS-Pro) and Loop-Swap 1 (LS-1) with comparisons to AT-III and P1-Arg-AAT:

Enzyme	Inhibitor k_{ass} ($M^{-1}s^{-1}$)			
	LS-Pro	LS-1	P1-Arg-AAT	AT-III (no heparin)
Trypsin		$(2.76\pm0.02) \times 10^6$	2.2×10^6	2.0×10^5
Thrombin	3.1×10^5	$(2.85\pm0.02) \times 10^4$	3.6×10^5	1.1×10^4
Factor Xa		$(3.76\pm0.03) \times 10^4$	2.1×10^4	2.8×10^3
Plasmin		$(2.34\pm0.05) \times 10^3$	8.5×10^5	1.9×10^3
Protein Ca	4.9×10^2	7.6 ± 0.7	4.9×10^4	0.14
t-PA		1.3 ± 0.2	1.4×10^2	

CLAIMS

1. A serine protease inhibitor protein which:
   (a) is substantially incapable of inhibiting activated protein C;
   (b) does not require activation by heparin; and
   (c) comprises a target sequence capable of interacting with the proteolytic active site of thrombin thereby to inhibit the proteolytic activity of thrombin.

2. A protein as claimed in claim 1, in which the target sequence comprises a sequence which is a reactive loop of a naturally occurring serpin, or a fragment thereof, or is substantially homologous to such a sequence.

3. A protein as claimed in claim 2, wherein the target sequence is derived from the reactive loop of antithrombin-III (AT-III).

4. A protein as claimed in claim 2 or 3, wherein the target sequence comprises at least residues P2, P1 and P1' of the naturally occurring serpin.

5. A protein as claimed in claim 3, wherein the target sequence comprises at least the residues Pro-Arg-Ser from the following sequence:
Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-Pro-Arg-Ser-Leu-Asn
or a fragment, homologue or mutant thereof.

6. A protein as claimed in any one of claims 1 to 5, which comprises, in addition to the target sequence, a base protein moiety, which comprises one or more sequences of a naturally occurring serpin.

7. A protein as claimed in claim 6, wherein the base protein moiety comprises enough of the naturally occurring serpin to ensure an essentially irreversible inhibition of thrombin.

8. A protein as claimed in claim 6 or 7, in which the naturally occurring serpin on which the base protein moiety is based is α_1-antitrypsin (AAT).

9. A mutein of α_1-antitrypsin (AAT) having one of the sequences shown in the following table, in which a dash indicates a residue which is the same as the corresponding residue of AAT and in which the sequences of AAT and antithrombin-III are given for comparative purposes only:

	P-12	*11*	*10*	*9*	*8*	*7*	*6*	*5*	*4*	*3*	*2*	*1*	*1'*	*2'*	*3'*	*4'*	*5'*	*6'*	*7'-P'*
AAT	**A**	**A**	**G**	**A**	**M**	**F**	**L**	**E**	**A**	**I**	**P**	**M**	**S**	**I**	**P**	**P**	**E**	**V**	**K**
Loop Swap I	-	-	A	S	T	A	V	V	I	A	G	R	S	L	N	-	-	-	-
Loop Swap II	-	-	-	-	-	A	V	V	I	A	G	R	S	L	-	-	-	-	-
Loop Swap III	-	-	-	-	-	A	V	V	I	A	G	R	S	L	N	-	-	-	-
LS-Pro	-	-	-	-	-	A	V	V	I	A	P	R	S	L	N	-	-	-	-
P' Swap 1	-	-	-	-	-	-	-	-	-	-	-	R	S	L	N	-	-	-	-
P' Swap 2	-	-	-	-	-	-	-	-	-	-	-	R	T	L	L	-	-	-	-
P3' Asn	-	-	-	-	-	-	-	-	-	-	-	R	-	-	N	-	-	-	-
P3' Lys	-	-	-	-	-	-	-	-	-	-	-	R	-	-	K	-	-	-	-
P3' Arg	-	-	-	-	-	-	-	-	-	-	-	R	-	-	R	-	-	-	-
AT-III	**A**	**A**	**A**	**S**	**T**	**A**	**V**	**V**	**I**	**A**	**G**	**R**	**S**	**L**	**N**	**P**	**N**	**P**	**N**

10. A protein as claimed in any one of claims 1 to 9 which has one or more (and preferably all) of the following association rate constants (k_{ass}, in $M^{-1}s^{-1}$):

1. with thrombin: at least 10^3, preferably at least 10^4, and optimally at least 5×10^4;

2. with activated protein C: no more than 10^3, preferably less than 10^2, and optimally below 10;

3. with tPA: less than 1.4×10^2.

11. A protein as claimed in any one of claims 1 to 10 for use in medicine, for example as an antithrombotic and/or anticoagulant agent.

12. The use of a protein as claimed in any one of claims 1 to 11 in the preparation of an antithrombotic and/or anticoagulant agent.

13. The use of a protein as claimed in any one of claims 1 to 11 in the preparation of an agent for the treatment or prophylaxis of disseminated intravascular coagulation (DIC), unstable angina, myocardial infarction, thrombotic stroke, thrombosis, pulmonary embolism or other blood clotting disorders.

14. A pharmaceutical or veterinary composition comprising one or more proteins as claimed in any one of claims 1 to 11 and a pharmaceutically or veterinarily acceptable carrier or excipient.

15. A nucleic acid (which may be RNA or, preferably, DNA) which:

(a) has a sequence coding for a protein as claimed in any one of claims 1 to 12, or its complementary strand;

(b) is substantially homologous with, or hybridises under stringent conditions to, either of the sequences in (a); or

(c) would be substantially homologous with, or would hybridise under stringent conditions to, a sequence in (a), but for the degeneracy of the genetic code.

16. Nucleic acid as claimed in claim 15, which is form of a vector.

17. A host cell transfected or transformed with a vector as claimed in claim 16.

18. Nucleic acid as claimed in claim 15, which is in the form of a transgenic expression construct.

19. A transgenic non-human animal whose germline contains nucleic acid as claimed in claim 18.

20. A transgenic animal as claimed in claim 19, which is a placental mammal and in which the transgene expression construct contains a promoter, operatively coupled to nucleic acid encoding a protein of any one of claims 1 to 12, which directs mammary gland expression of the protein into the mammal's milk.

21. A protein as claimed in any one of claims 1 to 11, which is detectably labelled.

22. A serine protease inhibitor protein which, in the absence of Heparin or Heparin-like substance:

(a) has an association rate constant (k_{ass} in $M^{-1}s^{-1}$) with thrombin of at least 5×10^4;

(b) has a difference in association rate constant

between thrombin and activated protein C of more than 350; and

(c) comprises a target sequence capable of interacting with a proteolytic active site of thrombin, thereby to inhibit the proteolytic activity of thrombin.

23. A protein as claimed in claim 22, modified by any one or more of the features of claims 2 to 13.

24. The use of a mutein of Antithrombin III (ATIII) comprising the following sequence:

P-7	6	5	4	3	2	1	1'	2'	3'
A	V	V	I	A	P	R	S	L	N

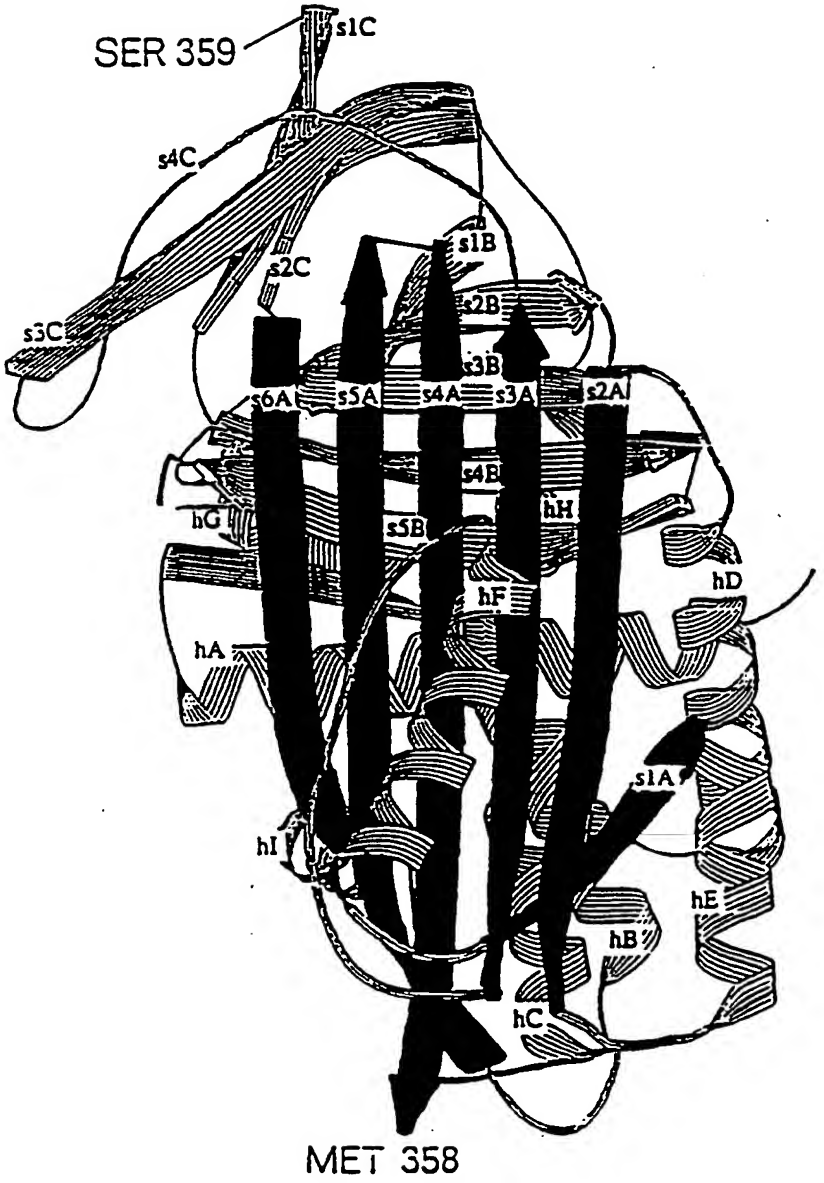
in the preparation of an antithrombotic and/or anticoagulant agent.

25. The use of a mutein of Antithrombin III (ATIII) comprising the sequence:

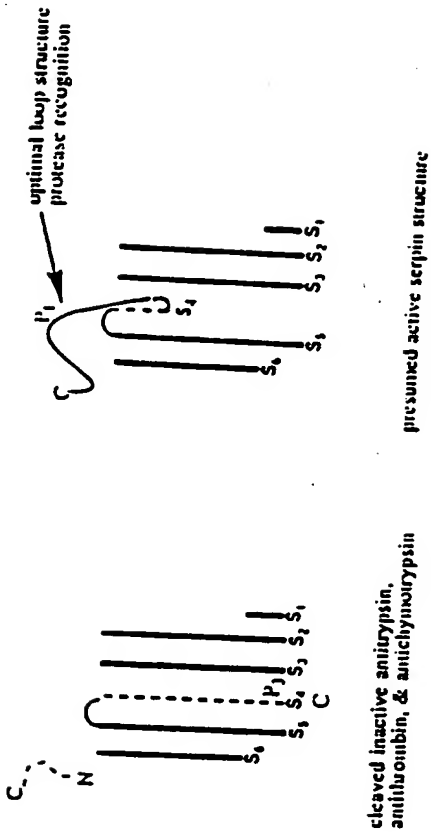
P-7	6	5	4	3	2	1	1'	2'	3'
A	V	V	I	A	P	R	S	L	N

in the preparation of an agent for the treatment or prophylaxis of disseminated intravascular coagulation (DIC), unstable angina, myocardial infarction, thrombotic stroke, thrombosis, pulmonary embolism or other blood clotting disorders.

FIGURE 1



α_1-AT



presumed active serpin structure

cleaved inactive antitrypsin, antithrombin, & antichymotrypsin

FIGURE 3

FIGURE 2

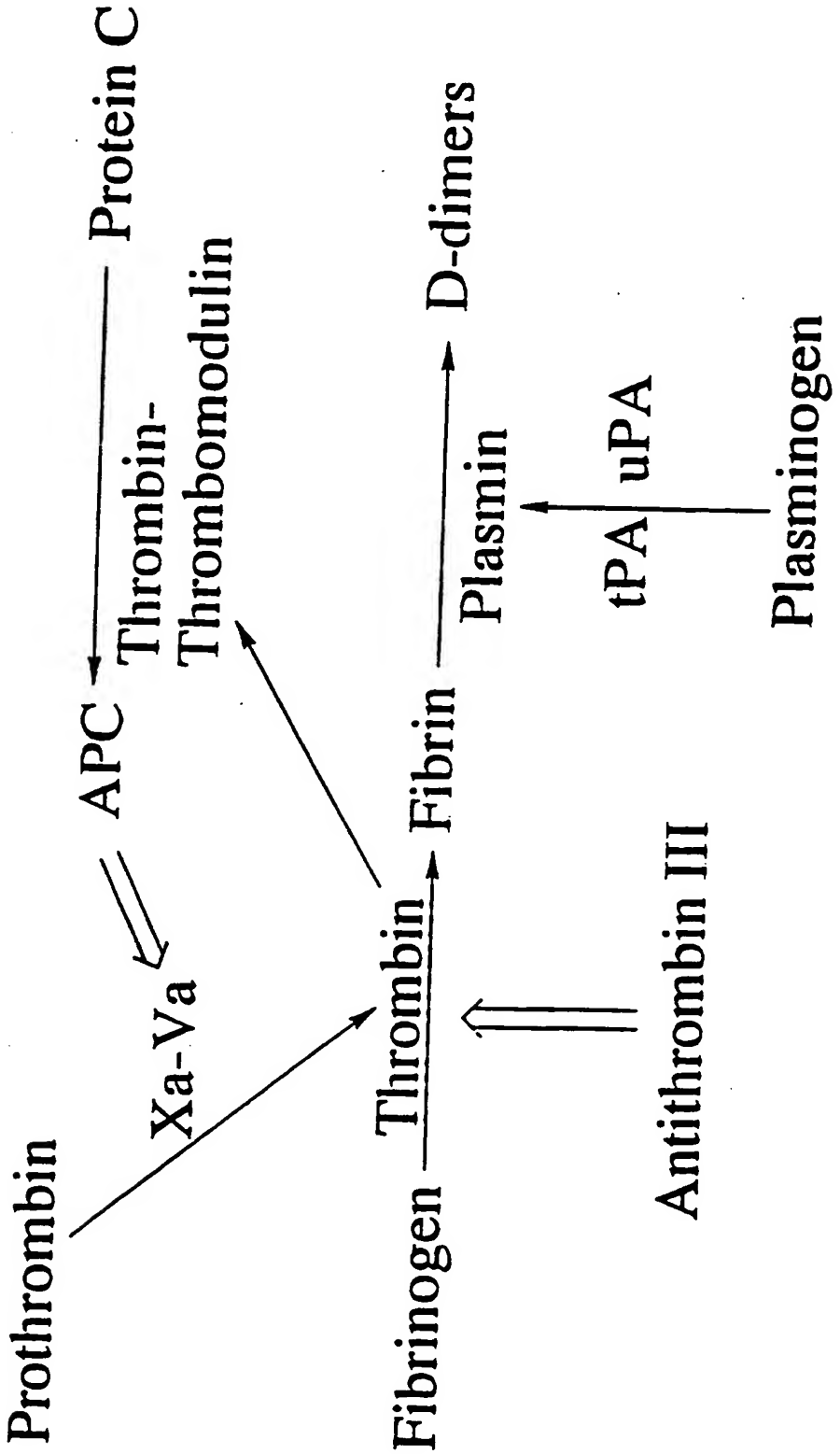

Protein C
APC
Thrombin-
Thrombomodulin
D-dimers
Plasmin
tPA
uPA
Plasminogen
Prothrombin
Xa-Va
Thrombin
Fibrin
Fibrinogen
Antithrombin III

FIGURE 4

FIGURE 5

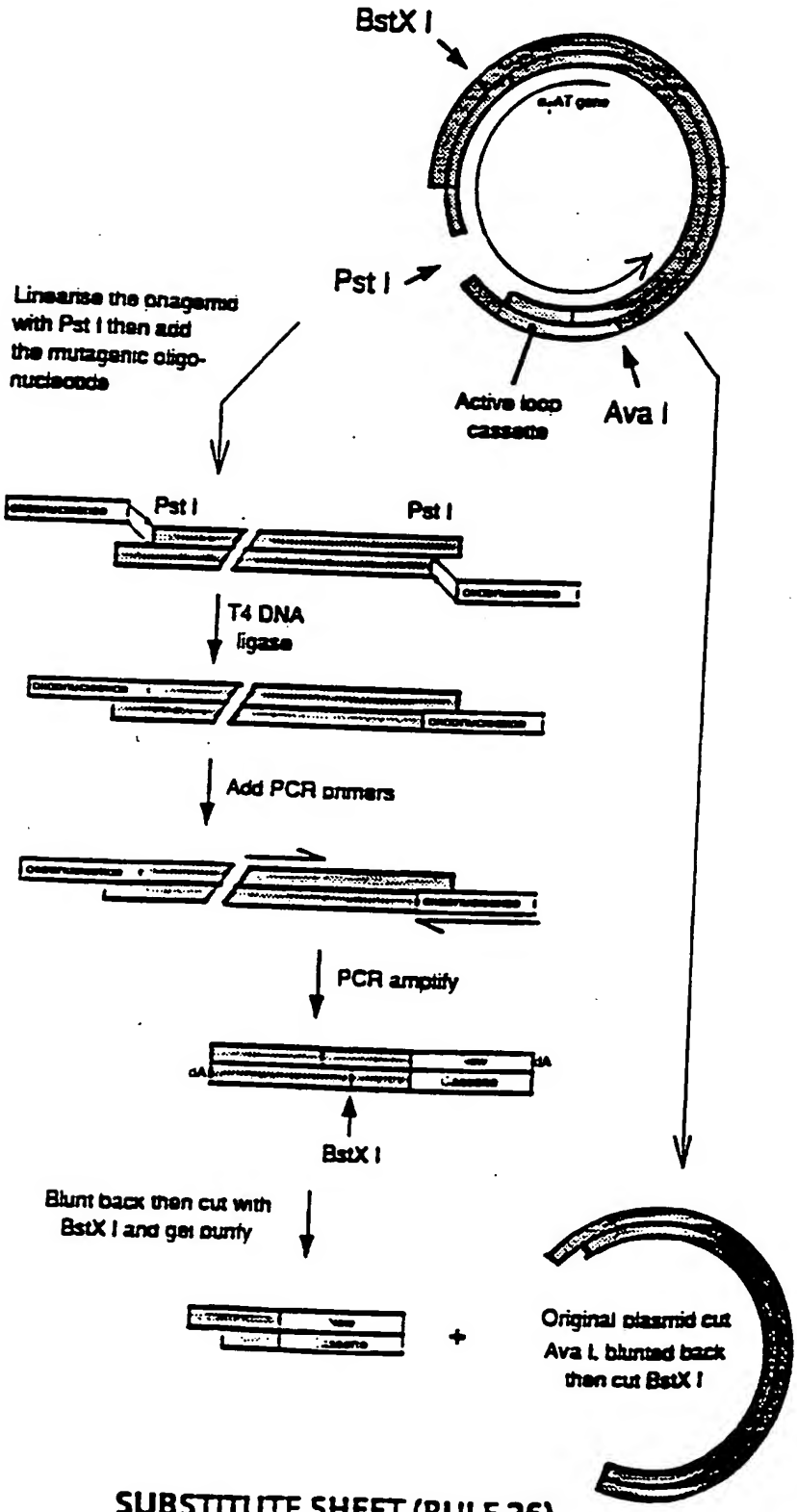
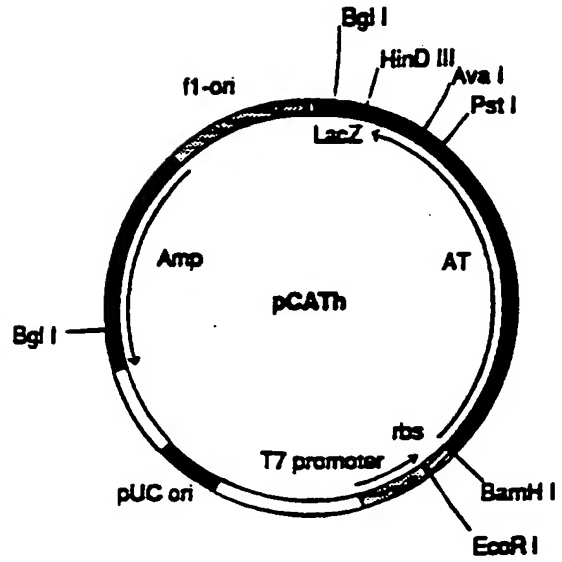

BstX I
Pst I
Linearise the phagemid with Pst I then add the mutagenic oligo-nucleotide
Active loop cassette
Ava I
Pst I
Pst I
T4 DNA ligase
Add PCR primers
PCR amplify
BstX I
Blunt back then cut with BstX I and gel purify
+
Original plasmid cut Ava I, blunted back then cut BstX I

FIGURE 6



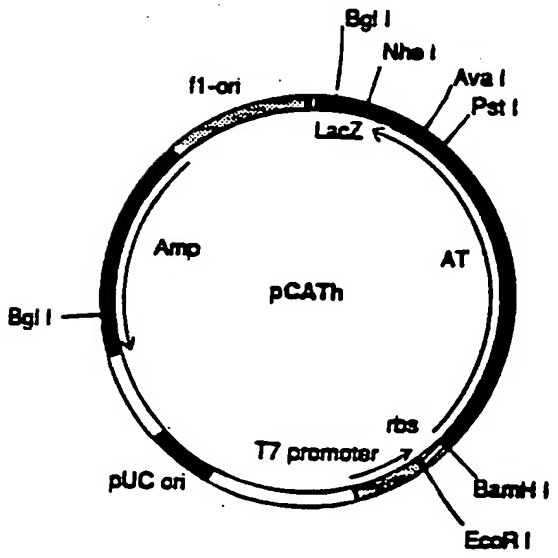
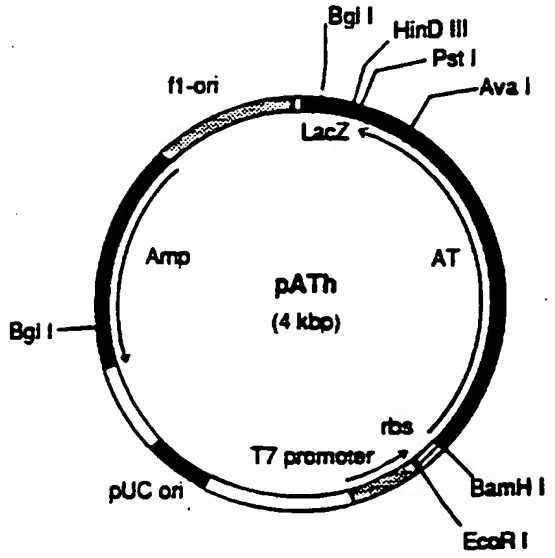


FIGURE 7

FIGURE 8



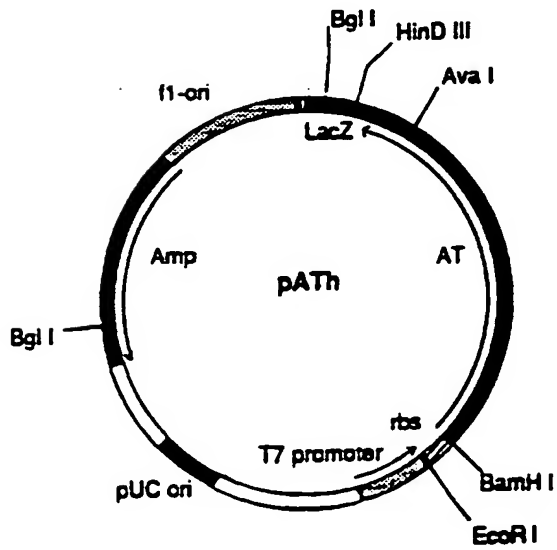


FIGURE 9

FIGURE 10

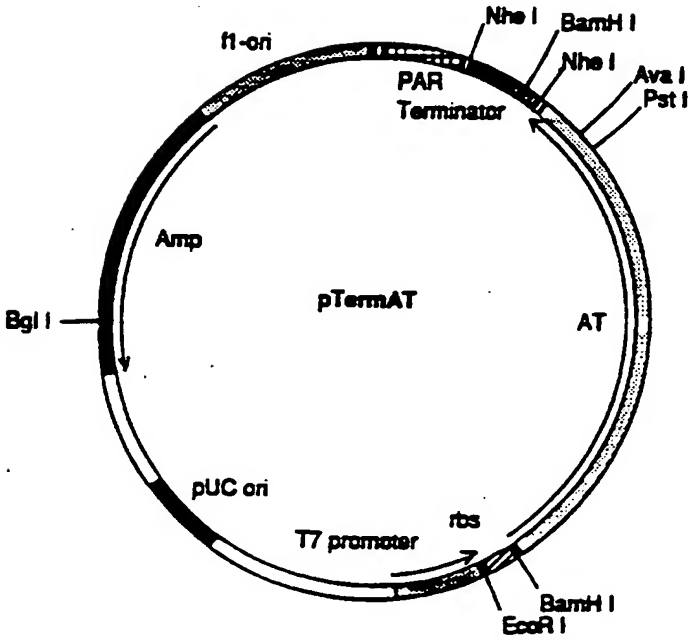
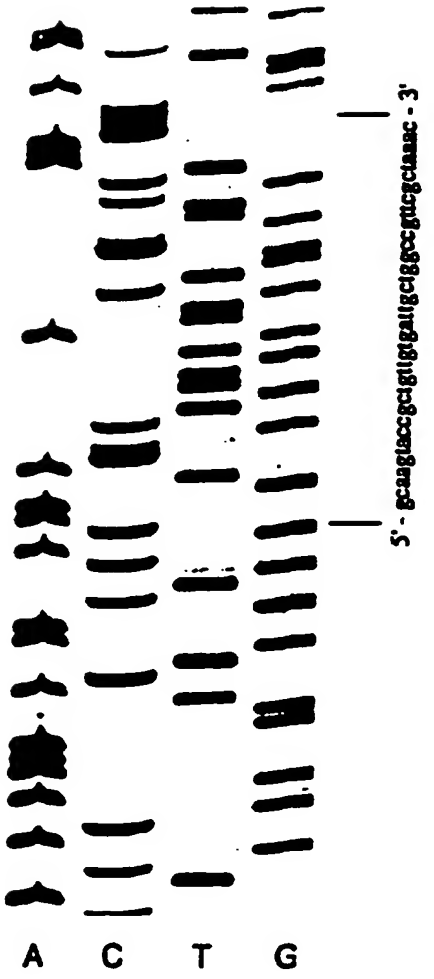

Nhe I
BamH I
f1-ori
Nhe I
Ava I
PAR
Terminator
Pst I
Amp
pTermAT
Bgl I
AT
pUC ori
rbs
T7 promoter
BamH I
EcoR I

FIGURE 11



INTERNATIONAL SEARCH REPORT

Inte: onal Application No
PCT/GB 95/02155

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C07K14/81 A61K38/57 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 568 833 (EISAI CO LTD) 10 November 1993 see page 2, line 12 - page 4, line 29 see page 8, line 8 - line 16 see tables 3,4 see page 13, line 43 - page 18, line 37 --- -/--	1-7, 10-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1996	27.02.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLOOD, vol. 73, no. 2, February 1989 pages 490-496, GEORGE ET AL 'CHARACTERIZATION OF ANTITHROMBINS PRODUCED BY ACTIVE SITE MUTAGENESIS OF HUMAN ALPHA1-ANTITRYPSIN EXPRESSED IN YEAST' see page 490,summary see page 490, paragraph 1 - page 2 see figure 1 see page 493, paragraph 2 - page 495, paragraph 4 ---	1-23
A	EP,A,0 169 114 (TRANSGENE SA) 22 January 1986 ---	
A	FR,A,2 599 752 (TRANSGENE SA) 11 December 1987 ---	
A	WO,A,91 00291 (AKZO NV) 10 January 1991 ---	
A	EP,A,0 238 473 (MONSANTO CO) 23 September 1987 ---	
A	EP,A,0 155 188 (ZYMOGENETICS INC) 18 September 1985 ---	
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A	PROTEIN ENGINEERING, vol. 1, no. 1, 1986 pages 29-35, JALLAT ET AL 'ALTERED SPECIFICITIES OF GENETICALLY ENGINEERED ALPHA1 ANTITRYPSIN VARIANTS' ---	
A	BIOCHEMISTRY, vol. 22, no. 22, 25 October 1983 pages 5055-5060, CHANDRA ET AL 'SEQUENCE HOMOLOGY BETWEEN HUMAN ALPHA1-ANTICHYMOTRYPSIN, ALPHA1-ANTITRYPSIN, AND ANTITHROMBIN III' see figure 6 --- -/--	

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INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/GB 95/02155

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 20, 19 May 1995 pages 11866-11871, HOPKINS ET AL 'DEVELOPMENT OF A NOVEL RECOMBINANT SERPIN WITH POTENTIAL ANTITHROMBOTIC PROPERTIES' see the whole document -----	1-25

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 95/02155

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		WO-A-	8600337	16-01-86
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		AU-B-	593766	22-02-90
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		JP-A-	61012289	20-01-86
		JP-A-	6105689	19-04-94